the lungs or liver, respectively. The delivery of plasmid DNA encoding several human cytokine genes,[50] i.e., IL-15 with Flt-3 ligand (Flt3L) or macrophage CSF, by hydrodynamic injection robustly induces the development of human monocytes/Mϕ in various organs. In addition, in a novel mouse strain in which the TPO gene was replaced with the human homolog, the development of total human myeloid lineage cells was significantly improved.[40] Accordingly, the frequency of monocytes was increased in the blood, but not in the BM. In our studies, the transgenic expression of hIL-3 and GM-CSF genes in NOG (hGM-CSF/IL-3 Tα NOG) mice also improved the development of whole human myeloid cells, including $CD14^+$ monocytes (Ito *et al.*, manuscr. submitt.).

DCs. In conventional humanized mice, several reports have demonstrated the presence of both myeloid DCs ($CD11c^+HLA\text{-}DR^+CD40^+CD86^+$) and plasmacytoid DCs ($CD123^+HLA\text{-}DR^+BDCA2^+$) in the BM, spleen and liver.[14,50] These $CD11c^+$ or $CD123^+$ DCs were functional, as demonstrated by their ability to induce activation of allogeneic human T cells or to produce interferon-α after stimulation.[14] The frequency of these cells, however, is generally low in the spleen (typically less than 1% in our studies). Chen *et al.*[50] demonstrated that the administration of plasmid DNA encoding human IL-4/GM-CSF/Flt3L or IL-5/Flt3L markedly increased the DC yield.[50] Enhancements in DC development were also observed in our hGM-CSF/IL-3 Tα NOG mice, in terms of both number and frequency, in various lymphoid organs (Ito *et al.*, manuscr. submitt.).

Granulocytes. Although granulocytes comprise a large fraction of human leukocytes, their frequency in humanized mice is very low (less than 2%–3% of human leukocytes in PB and BM in our studies). To improve differentiation of this population, several groups have attempted to produce novel humanized mouse strains by providing human cytokines. Billerbeck *et al.*[52] created a transgenic NSG strain that expressed the human stem cell factor, GM-CSF and IL-3 genes, and demonstrated a slight increased development of human $CD15^+$ granulocytes in the BM. In TPO KI mice, as mentioned above, a large number of human $CD66b^+$ granulocytes were produced in the BM.[40] Moreover, this strain enabled the development of mature human neutrophils with lobulated nuclei, which has not been achieved before. Additionally, in our hGM-CSF/IL-3 Tα NOG mice, we confirmed significant increases in $CD66b^+$ granulocyte numbers in the BM and PB. Furthermore, the presence of human basophils and eosinophils in the PB was detected by May–Giemsa staining (Ito *et al.*, manuscr. submitt.). This is the first report to demonstrate the development of these cell populations in humanized mice. Collectively, the development of human granulocytes in humanized mice has been greatly improved by the addition of human cytokines.

Mast cells. Mast cells play an important role in allergic responses by releasing intracellular granules containing histamine or various leukotrienes. Crosslinking of their surface Fc-epsilon receptor (FcεR) by IgE triggers a series of reactions.[53,54] Although there is little evidence suggesting the development of human mast cells, Kambe *et al.*[55] demonstrated the presence of human mast cells in the skin, spleen and BM of humanized NOG mice. These cells were positive for c-kit and CD203c, but expression of FcεR was not determined. Recently, we detected FcεR positive mast cells in the BM, spleen and several non-lymphoid tissues of hGM-CSF/IL-3 Tα NOG mice (Ito *et al.*, manuscr. submitt.). These data suggest that IL-3 and/or GM-CSF are important for inducing the differentiation of human mast cells.

HUMANIZED MODELS

Cancer

Due to their supply by the Central Institute for Experimental Animals, NOG mice have been predominantly used in this field. The characteristics of NOG mice include rapid growth of tumors and well-maintained characteristics after multiple passages. In a study by Machida *et al.*,[56] 100 HeLa S3 cells could be successfully engrafted in NOG mice; in contrast, 10^5 and 10^6 cells were required for engraftment in NOD/SCID and C.B-17-SCID mice, respectively. However, primary human tumors do not always engraft in NOG mice, even though these animals show higher engraftment than conventional immunodeficient mice. Some tumors, such as prostate carcinoma, which are difficult to engraft in SCID and nude mice, are also difficult to engraft in NOG mice. The growth of some tumor cell lines appears to be less than in conventional immunodeficient mice. The reason for this is unclear, but it may be explained by the differential adaptation of cell lines to conventional immunodeficient mice. *IL-2Rγ* gene inactivation may influence the growth of some tumors in NOG mice. Another characteristic of NOG mice is a high occurrence of metastasis. Genes responsible for metastasis have been investigated through the use of this characteristic.[57] The high homing capacity of human cells also appears to be maintained in NOG mice. When U266 myeloma cells were intravenously injected into NOG mice, they grew only in the BM, resulting in paralysis.[58]

Various cancer models have been established using these advantages.[51,59] On the other hand, a model that can be used to investigate immune responses to tumors has only recently been developed. Mismatching of HLA between tumor cells and hematopoietic cells from HSCs of different donors may cause severe GVHD or a lack of response. To induce an effective immune response against tumors in mice, HLA matching is required. Recently, Shultz *et al.*[42] reported that antigen-specific cytotoxic T lymphocytes were successfully induced in a newly established HLA class I (A-2) transgenic NSG mouse model by transfer of HLA-matched HSC. The development of these mice may lead to new immunotherapy models for cancer. The injection of human PBMCs (hPBMCs) into immunodeficient mice is known to cause severe GVHD; this provides a good model of GVHD.[60] We recently found that NOG-I-$A_\beta{}^{null}\beta2m^{null}$ mice showed mild GVHD, although high engraftment rates were observed as compared with non-transgenic NOG mice after transfer of hPBMCs (unpubl. data). Cotransplantation of a patient's tumor and hPBMCs into such immunodeficient mice may facilitate analysis of the immunological responses to the tumor.

Infectious diseases

Human lymphocytes, including T and B cells, predominantly develop in humanized mice transferred with HSCs. Therefore, appropriate models are provided for viruses that specifically infect lymphocytes and express their pathology, such as HIV-1, HTLV-1 and EBV. HIV-1 infection models have been widely used for the analysis of disease mechanisms and the development of anti-HIV-1 drugs,[61] as HIV-1 infects human T cells in *SCID-hu* mice.[26,62,63] This research is further accelerated through the use of HSC-transplanted immunodeficient mice, in which multilineage hematopoietic cells can be differentiated.[64–67] In this field, a unique model for HIV-1[68–70] reported by Garcia's group at the University of North Carolina and termed bone marrow–liver–thymus (BLT) mice, has attracted attention. As the name suggests, this model is generated by transplantation of fetal bone marrow, liver and thymus into a subcutaneous region of the kidney. The most attractive feature of BLT mice is reconstitution of human

mucosal immunity; this has not yet been obtained in human immune system mice transferred with HSCs. The human mucosal lymphoid apparatus, including Peyer's patches and gut-associated lymphoid tissue, has been successfully reconstituted in BLT mice, resulting in the development of mucosal immunity. They reported that the *IL-2Rγ* gene was indispensable for development of the mucosal lymphoid system, indicating that mucosal immunity cannot develop in NOG/NSG and BRG mice that contain a mutant *Il2rg* gene (reported at the Third International Workshop of Humanized Mice (IWHM 2011) held in Pittsburgh in October 2011).

This mouse appears to provide a better HIV-1 model as compared with conventional humanized mice transferred with HSCs. However, this model cannot be investigated from the aspect of humoral immunity involving B cells, and cannot be used in some countries such as Japan because of ethical issues. Additional genetic modifications of current immunodeficient mice may be necessary to overcome this disadvantage.

EBV usually presents in healthy subjects as a latent infection; however, it expresses a variety of pathological features in the healthy, termed EBV-associated infectious mononucleosis, hemophagocytic lymphohistiocytosis, lymphoproliferative disease, Burkitt's lymphoma and Hodgkin's disease in those immunosuppressed, due to HIV-1 infection or BM transplantation.[71] Since the report of EBV-associated lymphoproliferative disease by Traggiai *et al.*[14] using humanized BRG mice, various humanized mouse models of these clinical pathologies have been reported.[72–74]

Humanized models of tuberculosis, salmonellosis, yellow fever and Dengue fever have been investigated.[75–77]

Animal models appropriate for developing a malaria vaccine are eagerly desired, as malaria is one of the most common infectious diseases worldwide.[78] An interesting human malaria model uses immunodeficient mice with transplanted human liver. By injecting human hepatocytes into liver-damaged immunodeficient mice,[79–82] human hepatocytes replace the mouse hepatocytes. In these hu-liver mice, intrahepatic multiplication of *Plasmodium falciparum* has been observed.[83] However, human erythrocytes from human blood must be successively injected into the mice intraperitoneally, because human erythrocytes cannot develop from HSCs.[84] To establish the complete malaria life cycle in mouse models, it is necessary to develop mice in which human erythrocytes persist and flow in mouse peripheral blood. Hu-liver mice provide a good infection model for viruses specific to hepatocytes, including hepatitis C and B viruses.[85–87]

These models provide invaluable tools for analyzing the mechanisms of human infection and for developing chemotherapeutic agents such as antibodies.

GVHD

GVHD is a severe complication with a high mortality rate that often develops in patients who receive allogeneic BM transplantation for the treatment of acute/chronic leukemia, aplastic anemia or congenital immunodeficiency. Approximately 20 years ago, Mosier *et al.*[63] first demonstrated that the induction of xenogenic GVHD was possible in immunodeficient mice (C.B-17-SCID) by transplanting hPBMCs. In this model, the transplanted human T cells may be activated and attack the recipient mouse tissue, thus resulting in the development of allogeneic GVHD-like symptoms.

Although C.B-17-SCID or NOD/SCID mice have been useful in GVHD research, there are several problems. For example, human cell engraftment is relatively low, due to the mouse endogenous innate immune system. It also requires sublethal dose total body irradiation, which results in large variances in disease onset. Furthermore, a relatively large number of hPBMCs have to be administered intraperitoneally, but not intravenously, to induce the disease.[88] This does not reflect BM transplantation, where cells are infused intravenously. van Rijn *et al.*[89] used H-2^d-RAG2^{null} IL2rγ^{null} mice in which xeno-GVHD was induced by intravenous injection of hPBMCs; however, this model still depends on the infusion of large numbers of hPBMCs (3×10^7 cells/head) and total body irradiation. Our xeno-GVHD NOG mouse model has shown significant improvements over other models, such as the rapid onset of disease and uniform death of recipients. In addition, a smaller number of donor cells (2.5×10^6) is sufficient with intravenous injection, and total body irradiation is not always necessary.[60] These results were confirmed by other studies using NSG mice.[90]

Collectively, NOG or NSG mice are the most suitable platforms for basic and preclinical GVHD research at this time.

Humanized liver models

Humanized liver models, in which the mouse liver is replaced with a human liver, are useful for evaluating drug metabolism in the human liver, as there are numerous differences in liver enzymes between humans and mice. In the first human liver model developed by Mercer *et al.*,[81] SCID/*bg* mice carrying a urokinase-type plasminogen activator transgene (Alb-uPA) entered a profound hypofibrinogenemic state, which caused hepatocyte death. They transplanted human hepatocytes into the inferior splenic pole and demonstrated that human hepatocytes could be engrafted over 50% in the liver of these mice. To improve xenoengraftment of human hepatocytes, NOG-uPA[82] and FRG (fumarylacetoacetate hydrolase^{null}/RAG2^{null}/IL-2Rγ^{null})[79] mice, in which liver damage is induced by adenovirus-mediated uPA expression, were developed and showed markedly high rates of replacement by human hepatocytes (over 80%). Nevertheless, several problems limit their utility, such as poor breeding efficiency in the mouse colony, development of renal disease, and a very narrow time window for transplantation. Recently, Hasegawa *et al.*[80] established a novel NOG substrain that expresses the herpes simplex virus type 1 thymidine kinase (TK) transgene under the control of a mouse albumin promoter. Administration of ganciclovir, which is non-toxic to human and mouse tissues, ablated TK-expressing liver parenchymal cells. Herpes simplex virus type 1 TK NOG mice allowed high engraftment of human hepatocytes (over 80%) and did not develop systemic morbidity (liver disease, renal disease and bleeding diathesis) as seen in other uPA-dependent models. Stable, long-term humanization of TK NOG mice will facilitate studies of drug metabolism, toxicology and the virology of hepatitis viruses.

FUTURE PERSPECTIVES

Over the last 10 years, remarkable progress has been achieved in humanized mouse models using NOD/SCID/γc^{null}, Rag1/2^{null}γc^{null} mice, especially for hematology and immunology. Various humanized mouse models have been established that enable direct research of human diseases, which was previously impossible in immunocompetent animals. These models will also contribute to the analysis of mechanisms underlying human immune disorders and the development of vaccines against infectious diseases through the use of humanized mice that contain a wide variety of functional human hematopoietic cells.

However, several issues remain to be overcome, such as the rare differentiation of certain cell lineages from HSCs, immature differentiation and insufficient intercellular relationships. To overcome these problems, the inclusion of other immunodeficient mouse genes

or human genes responsible for cell differentiation and interaction has been investigated. These attempts may result in more appropriate immunodeficient humanized mice.

Recently, progress in the field of regenerative medicine has drawn our attention, following the establishment of human embryonic stem and inducible pluripotent stem cells. In the future, artificial human organs or HSCs developed from embryonic stem or inducible pluripotent stem cells may be available. Although these techniques have not yet impacted the field of humanized mice, new models will likely result from transplantation of artificial human organs and HSCs.

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Induction of human humoral immune responses in a novel HLA-DR-expressing transgenic NOD/Shi-scid/γc^{null} mouse

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Abstract

Mounting evidence has demonstrated that NOD-Shi/*scid*/γc^{null} (NOG) mice are one of the most suitable mouse strains for humanized mouse technologies, in which various human cells or tissues can be engrafted without rejection and autonomously maintained. We have characterized and analyzed various features of the human immune system reconstituted in NOG mice by transplanting human hematopoietic stem cells (hu-HSC). One of the problems of the quasi-immune system in these hu-HSC NOG mice is that the quality of immune responses is not always sufficient, as demonstrated by the lack of IgG production in response to antigen challenge. In this study, we established a novel transgenic NOG sub-strain of mice bearing the *HLA-DRA* and *HLA-DRB1:0405* genes, which specifically expresses HLA-DR4 molecules in MHC II-positive cells. This mouse strain enabled us to match the haplotype of HLA-DR between the recipient mice and human donor HSC. We demonstrated that T-cell homeostasis was differentially regulated in HLA-matched hu-HSC NOG mice compared with HLA-mismatched control mice, and antibody class switching was induced after immunization with exogenous antigens in HLA-matched mice. This novel mouse strain improves the reconstituted human immune systems that develop in humanized mice and will contribute to future studies of human humoral immune responses.

Keywords: adaptive immunity, HLA-DR, humanized mice, NOG mice, transgenic

Introduction

Recent advances in the development of novel mouse models that develop human hematopoietic systems have enabled the direct analysis of human hematopoiesis and immune responses with few constraints (1, 2). Such humanized mouse models are considered relevant for the study of basic human hematology and immunology as well as for translational research (3,4,5). For example, studies of leukemia stem cells largely depend on humanized mouse technology (6, 7). Some human-specific viral diseases, such as EBV or human T-cell leukemia virus-1 infection, can be also recapitulated in such mice (8,9,10,11). Furthermore, this technology makes it possible to evaluate the effects of therapeutic drugs on various human diseases (12,13,14).

The mouse strains NOD/Shi-scid/γc^{null} (NOG), NOD/LtSz-scid/IL-2Rγ$^{-/-}$ (NSG) and BALB/RAG-1 KO/γc KO (BRG) are the most suitable platforms for the *in vivo* reconstitution of human hemato-lymphoid systems, as the total deficiency of the endogenous murine immune systems in these mice enables long-term survival of various xenogenic grafts (1, 15). Mounting evidence has demonstrated that multiple lineages of human lymphocytes can develop *in situ* in these mice by transferring human hematopoietic stem cells (HSC) in the absence of other human-derived tissues (2, 16). Several groups have also examined the functionality of these quasi-human immune systems and have demonstrated that significant immune responses are possible in humanized mice (2, 15, 17). For example, human CD8$^+$ T cells in humanized mice exhibited effective cytotoxic activity and cleared infection with EBV (2). Additionally, human B cells produced antigen-specific IgM upon immunization with various

exogenous antigens (18, 19). However, such immunization could only induce few, if any, IgG responses (10, 17, 18). Poor IgG production suggests that the interactions between human B and T cells were not sufficient in these mice to activate the molecular machinery responsible for antibody class switching of the B cells. Thus, further improvements of the humanized mouse model are required for the reconstitution of a bona fide human immune system.

Our group previously showed that human T cells, particularly $CD4^+$ T cells, from conventional humanized mice (hu-HSC NOG) were unable to respond to antigenic stimulation *in vitro* to the same extent as normal human T cells from healthy donors do; the T cells from the hu-HSC NOG mice neither proliferated in response to anti-CD3 and anti-CD28 antibodies nor produced IL-2 (17). These human T cells were also susceptible to apoptosis (17, 20). These results raised the possibility that weak humoral responses in the hu-HSC NOG mice can be attributed, at least in part, to the suboptimal function of the human T cells. Although the precise cellular and molecular mechanisms involved in the impairment of these T cells are yet unclear, it is possible that T cells that are positively selected by mouse MHC in the mouse thymus are rendered anergic by the human antigen-presenting cells (APC) that express HLA in the periphery.

In this study, we established a novel transgenic NOG strain (NOG/HLA-DR4) that expresses the human *HLA-DRA* and *HLA-DRB1:0405* genes via a mouse MHC class II (mMHC II) promoter (21). We also generated NOG/HLA-DR4/I-Aβ KO mice that express the transgenic HLA-DR as the sole functional class II MHC so as to eliminate possible interference from mMHC II. Upon engraftment with HLA-DR:0405-positive HSC, but not with HLA-DR:0405-negative HSC, the humanized NOG/HLA-DR4/I-Aβ KO mice mediated effective humoral immune responses, as demonstrated by the accumulation of a significant amount of antigen-specific IgG in the sera after immunization. Our results indicate that HLA-restricted human immune responses could be provoked in this new NOG/HLA-DR4/I-Aβ KO strain. This new humanized mouse strain contributes to the study of the human immune system and the development of new drugs to manipulate human immune responses.

Methods

$CD34^+$ hematopoietic stem cells

The cord blood from full-term deliveries was obtained from the Miyagi Cord Blood Bank, following the institutional guidelines approved by the Tohoku University Committee on Clinical Investigations. Some $CD34^+$ cell samples were obtained from the RIKEN Bioresource Center Cell Bank (Tsukuba, Japan). HSC were isolated, as described elsewhere (17). Briefly, mononuclear cells were isolated using Lymphocyte Separation Medium (MP Biomedicals, Solon, OH, USA) after eliminating phagocytes with silica (Immuno Biological Laboratories, Takasaki, Japan). $CD34^+$ HSC were purified by magnetic cell sorting (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany). We used a biotin-conjugated anti-human CD34 mAb (Serotec, Oxford, UK), a blocking reagent for human Fc receptor (Miltenyi Biotech), and anti-biotin microbeads (Miltenyi Biotech) to label the cells and an AutoMACS pro separator (Miltenyi Biotech) to purify the labeled HSC. The typical purity of the $CD34^+$ fraction was >95%. The purified $CD34^+$ HSC were cryopreserved in Cell Banker (Juji Field, Tokyo, Japan) at –80°C in a deep freezer until use. The haplotype of the HLA-DRB1 locus of the cord blood samples was determined by HLA laboratory (Kyoto, Japan) to identify HLA-DRB1:0405-positive HSC. We collected HSC from 236 different donors in total and 38 individuals among them were positive for HLA-DRB1:0405.

Mice and reconstitution with human stem cells

Six-week-old female NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). To establish a transgenic strain of NOG mice that express human class II (HLA-DR4), we used the pDOI-5 vector (kindly provided by Drs Mathis and Benoist of Harvard Medical School, Boston, MA, USA) (21) so that the expression of the *HLA-DRA* and *HLA-DRB1:0405* genes were regulated by the mouse class II promoter. The DNA constructs were microinjected into fertilized eggs of NOD mice by conventional methods. The founder mice were screened for the expression of HLA-DR on mouse B cells by flow cytometric analysis. The HLA-DR-expressing transgenic NOG mice (NOG/HLA-DR4) were then crossed with NOG I-$A\beta^{-/-}$ mice (17, 22) to create NOG/HLA-DR4/I-$A\beta^{-/-}$ mice. All mice were maintained in the animal facility at Tohoku University School of Medicine under specific pathogen-free conditions, and all animal experiments were properly conducted according to institutional guidelines. NOG/HLA-DR4/I-$A\beta^{-/-}$ mice were irradiated with 120 cGy X-rays and grafted with 1×10^5 $CD34^+$ cells with the appropriate HLA-DR haplotype.

Antibodies and flow cytometric analysis

The following mAbs were used. To identify mouse cells, anti-I-A^k–FITC (also reactive for I-A^{g7} in NOD), anti-CD11c–PE, anti-CD11b–PE, anti-CD19–PE, anti-6C3–PE and anti-CD45–allophycocyanin (APC) were purchased from BD Pharmingen (San Jose, CA, USA). To identify human cells, anti-CD24–FITC and purified anti-HLA-DR were purchased from eBioscience (San Diego, CA, USA) and anti-CD19–FITC, anti-CD34–FITC, anti-CD45RA–FITC, anti-CD5–PE, anti-CD8–PE, anti-CD38–PE, anti-IL-4–PE, anti-IL-2–PE–Cyanine7(Cy7), anti-CD62L–PE–Cy7, anti-CD3–APC, anti-CD4–APC, anti-CD19–APC, anti-IFN-γ–APC and anti-CD45–APC–Cy7 were purchased from BD Pharmingen. Anti-CD4–Pacific Blue and anti-CD8–Pacific Blue were purchased from Biolegend (San Diego, CA, USA). Anti-CD34–biotin was purchased from AbD Serotec (Kidilington, UK).

To analyze human lymphocytes in hu-HSC NOG/HLA-DR4/I-$A\beta^{-/-}$ mice, multicolor flow cytometric analysis was performed using a FACS Canto II flow cytometer (BD Biosciences). Peripheral blood was obtained from the retro-orbital venous plexus through heparinized pipettes to periodically monitor reconstitution. At the time of sacrifice, single-cell suspensions were prepared from the spleen or bone marrow by conventional methods. The cells were stained with the relevant mAbs for 15 min on ice, then washed with cold PBS containing 2% FCS and stained with the appropriate secondary antibodies. We used Cytofix/Cytoperm solution

(BD Biosciences) for intracellular staining according to the manufacturer's instructions. After the final wash, the cells were subjected to flow cytometric analysis. The proportion of each cell lineage was calculated using FACS Diva software (BD Biosciences).

The ELISA

The concentration of human IgM and IgG in the sera of reconstituted NOG mice was measured using a human Ig assay kit (Bethyl, Denver, CO, USA). For the detection of ovalbumin (OVA)-specific human IgM and IgG antibodies, humanized NOG/HLA-DR4/I-Aβ$^{-/-}$ mice were immunized once a week for 4 weeks with an emulsion of 20 μg OVA whole protein (Bioresearch Technologies, Novato, CA, USA) with alum (Cosmo Bio, Tokyo, Japan) in a total volume of 100 μl by intra-peritoneal injection. Sera from the immunized mice were harvested 3 days after the final immunization. Specific antibodies against OVA were measured by a standard ELISA. Briefly, 96-well plates were coated with 10 μg ml^{-1} OVA at 4°C overnight. After washing and blocking with PBS containing 1% BSA, the collected serum samples were loaded. Sera were serially diluted three-fold with blocking solution. HRP-conjugated anti-human Ig antibody was used as a secondary antibody. Both anti-IgG-specific and anti-IgM-specific antibodies were purchased from Bethyl (Montgomery, TX, USA). *o*-Phenylenediamine was used as a substrate for detection. The absorbance at 450 nm was measured by a microplate reader. Serum titers were defined as the dilution at which the absorbance of the sample was equivalent in intensity to that of non-immunized mice (background signal).

Immunohistochemistry

Tissue samples were fixed with 4% PFA, dehydrated with graded alcohol and embedded in paraffin. After sectioning, the specimens were treated with heated citrate buffer and stained with mouse anti-HLA-DR antibody overnight at 4°C. Subsequently, endogenous peroxidase activity was quenched by incubating the specimen for 20 min with 0.3% hydrogen peroxide (H_2O_2) in methanol at room temperature. The samples were further incubated with labeled polymer (Dako EnVision System; Dako, Glostrup, Denmark) as a secondary reagent for 40 min at room temperature. Staining was completed after a 5- to 10-min incubation with 3.3′-diaminobenzidine plus the chromogen substrate. Hematoxylin was used for counter staining.

In vitro cultures

Single-cell suspensions of the spleen from hu-HSC NOG/HLA-DR4/I-Aβ$^{-/-}$ mice were prepared, as described above, ~16 to 24 weeks after reconstitution. For T-cell stimulation, total spleen cells of the mice were labeled with carboxyfluorescein succinimidyl ester (CFSE; Molecular probes, Eugene, OR, USA) according to a standard protocol. Then, the cells were seeded at 1 × 10^5 T cells per well in a 48-well plate and cultured with 10 μg ml^{-1} soluble anti-CD3 (clone OKT3; BD Pharmingen) and 1 μg ml^{-1} anti-CD28 (Biolegend) antibodies in 500 μl per well RPMI 1640 medium supplemented with 10% FCS and 50 μM 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan). The cells were collected on day 6, and T-cell proliferation was assessed by flow cytometry after staining with fluorochrome-conjugated anti-CD4 and anti-CD8 antibodies. To determine cytokine production, cells were stimulated with 50 ng ml^{-1} phorbol myristate acetate (PMA) and 1 μg ml^{-1} ionomycin for 4 h in the presence of Golgi plug (BD Pharmingen), and the accumulated intracellular cytokines were stained with the relevant mAbs according to the manufacturer's instructions, followed by flow cytometric analysis.

Results and discussion

To investigate whether the transgenic expression of human class II (HLA II) molecules in humanized NOG mice influenced the function of the reconstituted human immune system, we developed a novel transgenic strain, NOG/HLA-DR4, which expresses the *HLA-DRA* and *HLA-DRB1:0405* genes. This HLA haplotype comprises one of the highest frequencies (13.62%, according to the HLA laboratory; http://www.hla.or.jp/, as of 2010) in the Japanese population and increases the likelihood of identifying donor HSC with this HLA haplotype. The expression of the *HLA-DR* genes was under the control of the mMHC II promoter (21). We first examined whether mature HLA-DR molecules were properly formed and expressed on the mouse cell surface. Flow cytometric analysis revealed that a substantial number of mMHC II-positive APC [i.e. B cells, dendritic cells (DC) and macrophages (Mϕ)] in the immunosufficient NOD/HLA-DR4 transgenic mice expressed the HLA-DR protein at significant levels (Fig. 1A) and that the expressions of I-A and HLA-DR were well correlated (Supplementary Figure 1, available at *International Immunology* Online). Additionally, thymic epithelial cells (TEC), which are responsible for the positive selection of T cells, also expressed HLA-DR (Fig. 1A). Immunohistochemistry confirmed that HLA-DR-positive cells resided in the stromal area (Fig. 1B). APC (DC and Mϕ) from immunodeficient NOG/HLA-DR4/I-Aβ$^{-/-}$ mice also expressed HLA-DR (Fig. 1C). Collectively, the expression pattern of transgenic HLA-DR was consistent with that of mMHC II.

The NOG/HLA-DR4/I-Aβ$^{-/-}$ mice were reconstituted with HSC purified from cord blood. Both DRB1:0405-positive and negative HSC were used to compare the effects of matching, or not matching, the HLA haplotype. The chimerism of human hematopoietic cells in the bone marrow progressively increased over time, irrespective of the HLA haplotype of the donor HSC, and there were no significant differences between these groups (data not shown). The chimerism of human CD45$^+$ cells in the spleen was also comparable between these two groups (Supplementary Figure 2, available at *International Immunology* Online). Human B and T cells were detected in the spleen ~3 months after HSC transplantation, as seen in conventional NOG mice (Supplementary Figure 2, available at *International Immunology* Online), and the thymus was also colonized by human T cells, which indicated proper differentiation (Supplementary Figure 3, available at *International Immunology* Online). The number of thymocytes was not significantly influenced by the presence of HLA-DR transgene (Supplementary Figure 3, available at *International Immunology* Online). Our previous work demonstrated that the majority of human B cells in hu-HSC NOG

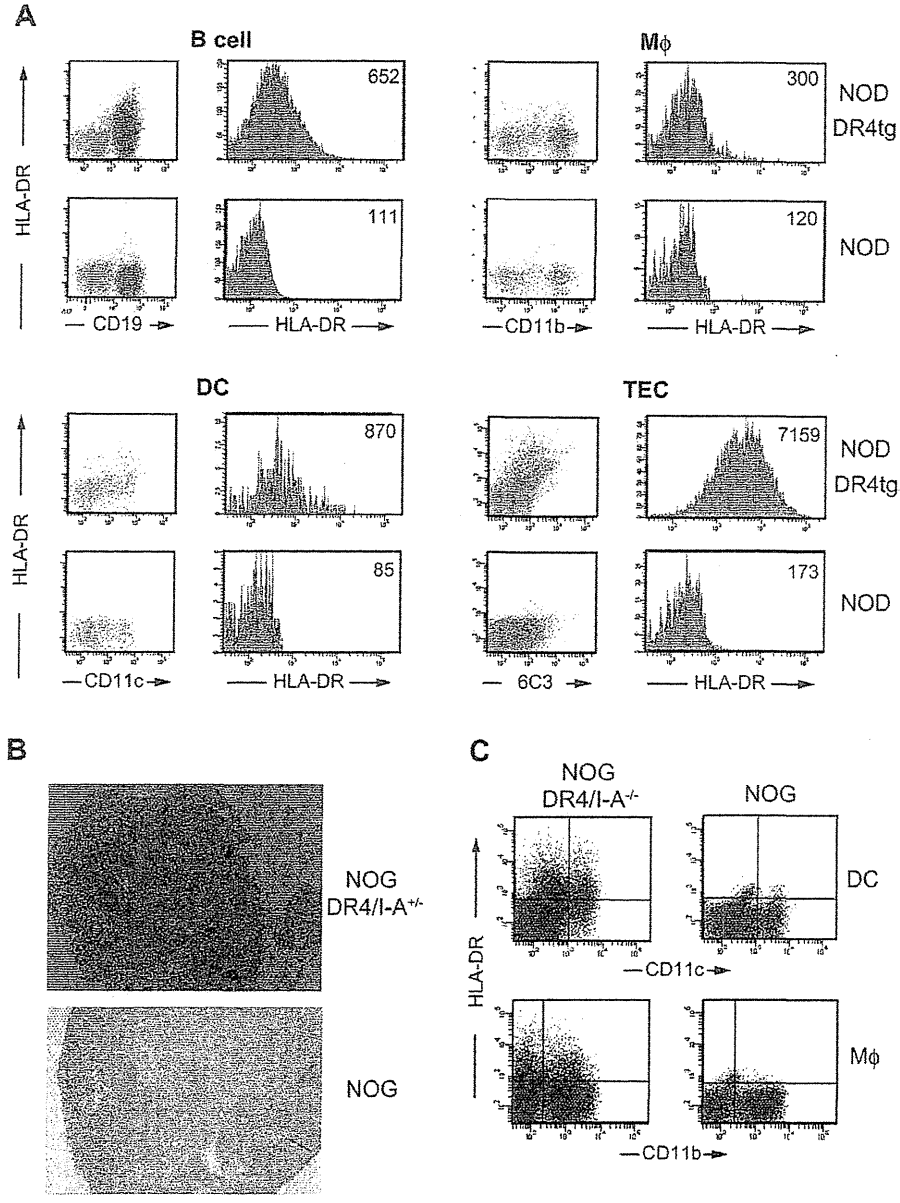

Fig. 1. Expression of HLA-DR in the transgenic mouse strain, NOG/HLA-DR4. (A) Expression of HLA-DR by various APC from immunosufficient NOD/HLA-DR4 (NOD/DR4) transgenic mice. Spleen cells from HLA-DR transgenic or non-transgenic NOD mice were stained with anti-HLA-DR antibody and anti-CD19 antibody for B cells, anti-CD11c antibody for DC, anti-CD11b antibody for Mϕ, or anti-6C3 antibody for TEC. The expression of HLA-DR in various APC subgroups (red plots) is shown in the left columns, with histograms in the right columns. Histogram values represent the mean fluorescent intensity. Representative flow plots are shown (n = 5). (B) Histological analysis of the thymus of a NOG/DR4 mouse. The thymi from hu-HSC NOG/DR4 I-$A^{+/-}$ mice or control hu-HSC NOG mice were isolated 20 weeks after reconstitution, fixed with 10% formalin and processed for immunohistochemical analysis for the expression of HLA-DR. Red arrows represent HLA-DR-positive TEC. Representative results are shown (n = 4). (C) Expression of HLA-DR in the APC of NOG/DR4. Spleen cells from HLA-DR transgenic or non-transgenic NOG mice were stained with anti-HLA-DR together with anti-CD11c or CD11b antibodies. Representative flow plots are shown (n = 4).

mice had immature phenotypes, which were similar to the transitional 1 B-cell phenotype of normal human B cells. Our analysis of human B cells in the hu-HSC NOG/HLA-DR4/I-A$\beta^{-/-}$ mice revealed that the introduction of HLA-DR did not influence the differentiation of human B cells in the transgenic NOG mice irrespective of the HLA haplotype of the donor HSC (Supplementary Figure 4, available at *International Immunology* Online).

There was no statistically significant difference in the total cellularity of the spleens from the DRB1:0405^{+} HSC-grafted mice versus the DRB1:0405^{-} HSC-grafted mice at various time points after reconstitution (Fig. 2A). The frequency and

the absolute number of $CD3^+$ splenic T cells were also compared between these two groups (Fig. 2B). At early time points (i.e. ~4 months) after HSC transplantation, the frequency of $CD3^+$ T cells was significantly higher in $DRB1{:}0405^+$ HSC-grafted mice than in $DRB1{:}0405^-$ HSC-grafted mice, though this difference became less evident at later time points (Fig. 2B). Accordingly, the absolute number of $CD3^+$ T cells was higher in the $DRB1{:}0405^+$ HSC-grafted mice than in the $DRB1{:}0405^-$ HSC-grafted mice 4–5 months after reconstitution, although the latter group reached a number of $CD3^+$ T cells equivalent to the former group by 6 months (Fig. 2B). The frequency and the absolute number of $CD4^+$ splenic T cells showed a similar pattern (Fig. 2C), in which $DRB1{:}0405^-$ HSC-grafted mice had higher numbers of $CD4^+$ T cells than $DRB1{:}0405^-$ HSC-grafted mice at 4 and 5 months posttransplantation. The analysis of $CD8^+$ T cells showed the mild increase of the proportion in $DRB1{:}0405^+$ HSC-grafted mice compared with that in $DRB1{:}0405^-$ HSC-grafted mice (Fig. 2D), which was in contrast with the prominent increase of $CD4^+$ T cells.

In accordance with the increase of $CD3^+$ T cells, there was a tendency that the frequency and the number of human B cells became lower in $DRB1{:}0405^+$ HSC-grafted mice than in $DRB1{:}0405^-$ HSC-grafted mice at 6 months (Supplementary Figure 4, available at *International Immunology* Online). This did not, however, reach statistical significance.

We next analyzed the composition of human $CD4^+$ T cells in the hu-HSC NOG/HLA-DR4/I-$A\beta^{-/-}$ mice in detail. There was a strikingly low frequency of $CD4^+CD45RA^+CD62L^{hi}$ naive T cells (T_{naive}) and a corresponding high frequency of $CD4^+CD45RA^-CD62L^{lo}$ effector memory-like T cells (T_{EM}) and $CD4^+CD45RA^-CD62L^{hi}$ central memory-like T cells (T_{CM}) in the humanized mice compared with normal human T cells from peripheral blood (Fig. 3A). The frequency of T_{EM} significantly increased in the $DRB1{:}0405^+$ HSC-grafted mice, but not in the $DRB1{:}0405^-$ HSC-grafted mice, over the course of reconstitution (Fig. 3B), while the absolute number of T_{EM} significantly increased in both groups. In contrast, T_{naive} in the $DRB1{:}0405^+$ HSC-grafted mice gradually decreased in number and frequency, although this did not reach statistical significance (Fig. 3B) due to large variance, which may be largely attributable to the variation in donor HSC. The analysis of T-cell sub-populations revealed the remarkable expansion of T_{EM} and the corresponding reduction of T_{naive} and T_{CM} in $DRB1{:}0405^+$ HSC-grafted mice, whereas the frequencies of these T-cell sub-populations were not significantly altered in the $DRB1{:}0405^-$ HSC-grafted mice (Fig. 3C). These results suggested that under HLA-matched conditions, human $CD4^+$ T_{naive} cells in NOG/HLA-DR4/I-$A\beta^{-/-}$ mice vigorously proliferated in an HLA-dependent manner and differentiated into T_{EM} cells.

We next examined the function of the reconstituted human immune system in hu-HSC NOG/HLA-DR4/I-$A\beta^{-/-}$ mice. NOG/HLA-DR4/I-$A\beta^{-/-}$ mice reconstituted with HLA-matched or HLA-mismatched HSC were immunized with OVA protein plus alum adjuvant. After four weekly immunizations, the sera were collected and the presence of OVA-specific antibodies was examined by ELISA (Fig. 4A). As expected, a large quantity of OVA-specific human IgM was detected in all the mice (Fig. 4B left). High levels of OVA-specific human IgG

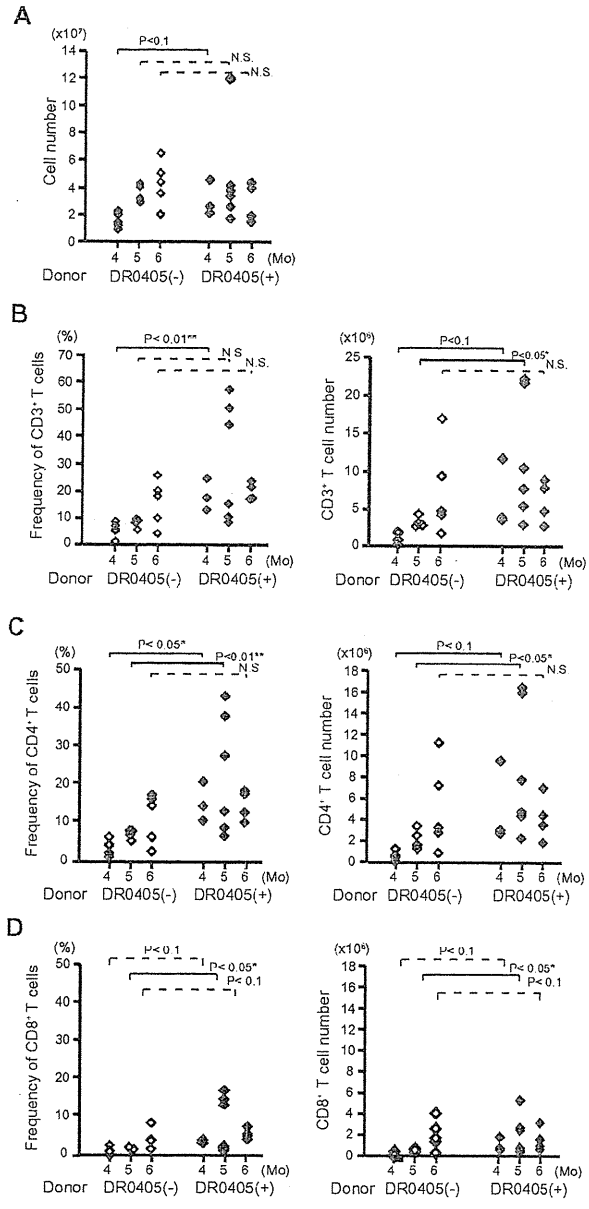

Fig. 2. Analysis of T cells in hu-HSC NOG/DR4/I-$A^{-/-}$ mice. (A) The number of splenocytes in hu-HSC NOG/DR4/I-$A^{-/-}$ mice after HSC transplantation. Spleen cells were isolated from humanized NOG/DR4/I-$A^{-/-}$ mice at various time points after transplant with HLA-matched ($DR0405^+$) or HLA-mismatched HSC ($DR0405^-$) HSC. The cells were enumerated and plotted ($n \geq 3$ per time point). Welch's *t*-test was used to calculate statistical significance. (B, C and D) Analysis of the frequency and cell number of T cells in hu-HSC NOG/DR4/I-$A^{-/-}$ mice. The splenocytes from humanized mice were stained with anti-CD3, anti-CD4 and anti-CD8 antibodies and analyzed with a flow cytometer. The frequencies of $CD3^+$ T cells (B), $CD4^+CD3^+$ T cells (C) and $CD8^+CD3^+$ T cells (D) in the spleen are plotted in the left panels. The absolute number of those populations (right panels) was obtained by multiplying frequency and total cell number.

were also detected in the sera of 6/10 immunized mice with HLA-matched HSC (Fig. 4B right). Considerable variance was observed among the mice, and the titer of OVA-specific

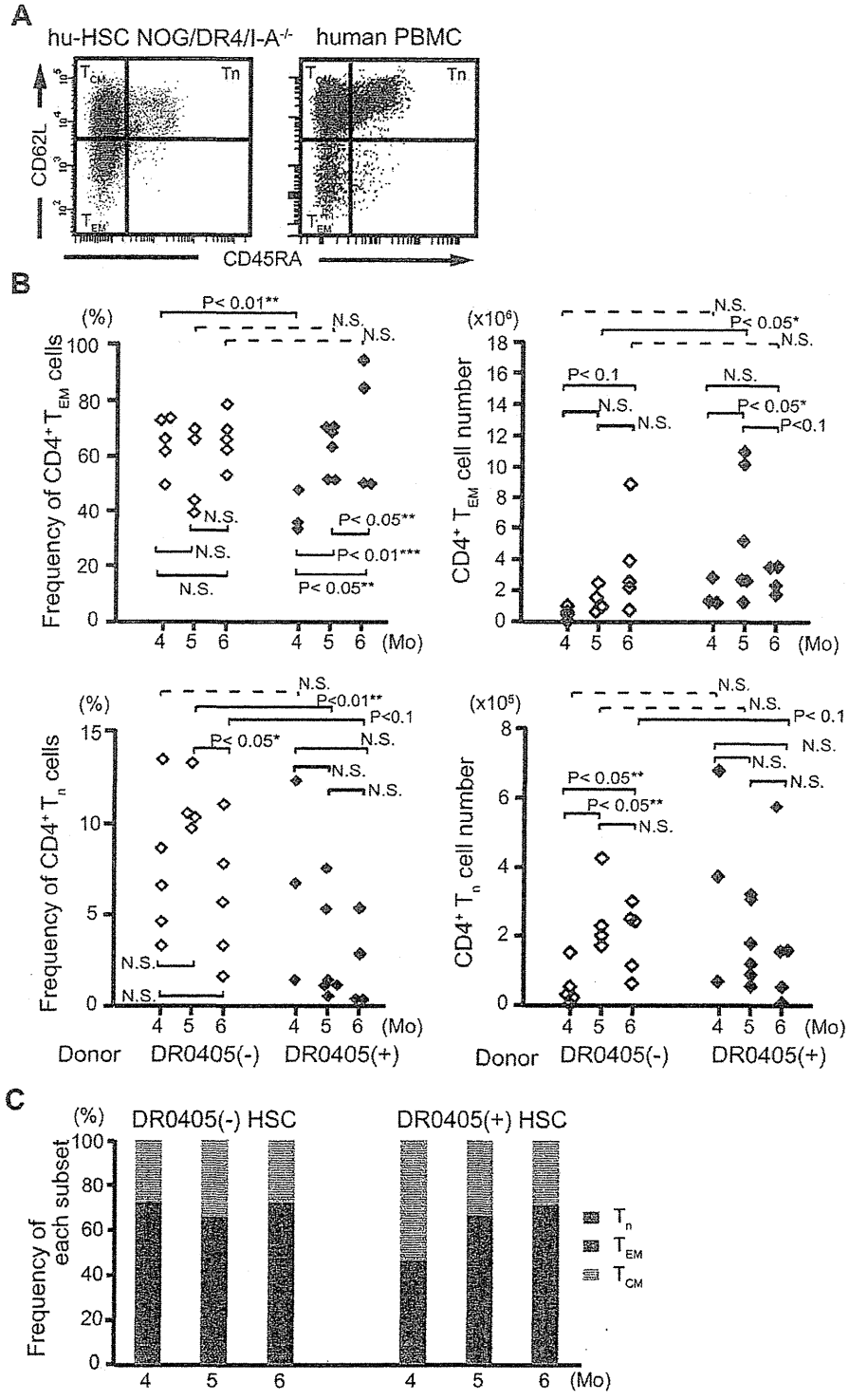

Fig. 3. Analysis of the CD4$^+$ T-cell sub-population in hu-HSC NOG/DR4/I-A$^{-/-}$ mice. (A) The expression pattern of CD45RA and CD62L in human CD4$^+$ T cells from humanized mice. Splenocytes from hu-HSC NOG/DR4/I-A$^{-/-}$ mice were isolated 20 weeks after HSC reconstitution and stained with anti-CD4, anti-CD45RA and anti-CD62L antibodies. Representative flow plots from hu-HSC NOG/DR4/I-A$^{-/-}$ mice and normal human peripheral blood are shown ($n > 10$). (B) The frequencies and the absolute cell numbers of T_{naive} and T_{EM} in hu-HSC NOG/DR4/I-A$^{-/-}$ mice. The frequencies (left panels) and absolute numbers (right panels) of CD45RA$^+$CD62L^{hi} T_{naive} (top) and CD45RA$^-$CD62L^{lo} T_{EM} (bottom) populations, which were obtained by flow cytometry, are shown (left panels). (C) The proportion of each CD4$^+$ T-cell sub-population in hu-HSC NOG/DR4/I-A$^{-/-}$ mice. The mean frequencies of CD45RA$^+$CD62L^{hi} T_{naive}, CD45RA$^-$CD62L^{lo} T_{EM} and CD45RA$^-$CD62L^{hi} T_{CM} populations are shown over time.

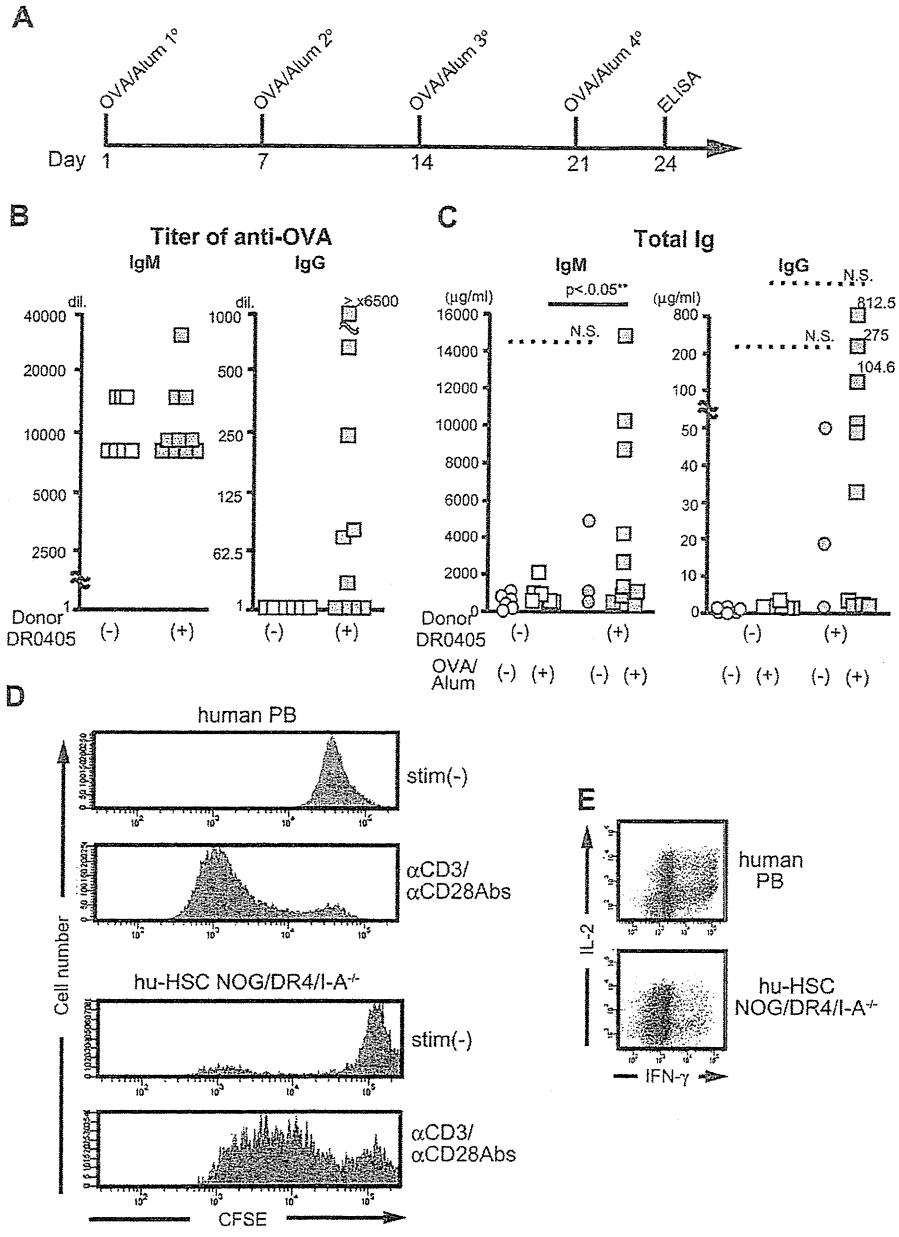

Fig. 4. Induction of humoral immune responses in hu-HSC NOG/DR4/I-A$^{-/-}$ mice. (A) Schematic protocol for OVA immunization. OVA (10 μg) and alum were injected intra-peritoneally into hu-HSC NOG/DR4/I-A$^{-/-}$ mice 20–25 weeks after HSC transplantation. After four weekly OVA immunizations, sera were collected and the presence of OVA-specific human IgM and IgG was examined by ELISA. (B) The production of anti-OVA antibody in the sera. The titers of OVA-specific IgM (left panel) and OVA-specific IgG (right panel) in sera from HLA-mismatched or HLA-matched HSC groups are shown. (C) The amount of total human IgM (left panel) and IgG (right panel) in sera. Sera were collected from HLA-mismatched (white symbols) or HLA-matched HSC groups (gray symbols) before (circles) and after (squares) the immunization with OVA and the Ig levels were quantified. (D) T-cell responses to stimulation with anti-CD3 and anti-CD28 antibodies. Splenocytes from hu-HSC NOG/DR4/I-A$^{-/-}$ mice with HLA-matched HSC were isolated 25 weeks after transplantation and compared with peripheral blood from a healthy human donor. Cells were labeled with CFSE and cultured *in vitro* in the presence of stimulating antibodies. At day 6, the cells were recovered and stained with anti-CD4. The dilution of CFSE was measured by flow cytometry. Representative flow plots are shown ($n > 10$). (E) Cytokine production by human CD4$^+$ T cells. *In vitro*-stimulated T cells from hu-HSC NOG/DR4/I-A$^{-/-}$ mice were re-stimulated with PMA/ionomycin in the presence of Golgi plug for 4 h at 37°C. The cells were fixed, permeabilized and stained with various anti-cytokine antibodies. Representative flow plots are shown ($n \geq 5$).

IgG in one mouse reached to >6250-fold higher than that of non-immunized control mice. OVA-specific IgG was not detected in hu-HSC NOG/HLA-DR4/I-Aβ$^{-/-}$ mice with HLA-mismatched HSC, although they showed OVA-specific IgM production (Fig. 4B). As for the total amount of IgM and IgG, there was no significant difference between the mice

with HLA-matched HSC and HLA-mismatched HSC before immunization (Fig. 4C, white symbols). After immunization, total IgM and IgG was increased in several mice in HLA-matched group, but none in HLA-mismatched group (Fig. 4C, gray symbols) and the increase was statistically significant for IgM, but not for IgG. These results suggested that humanized HLA transgenic mice with HLA-matched HSC transplantation mounted immune responses sufficient to induce antibody class switching in the human B cells.

We next analyzed T-cell function in the hu-DRB1:0405^{+} HSC NOG/HLA-DR4/I-A$\beta^{-/-}$ mice. The human T cells showed significant proliferation in response to anti-CD3 and anti-CD28 antibodies *in vitro*, and the activated T cells produced IL-2 and IFN-γ upon stimulation with PMA and ionomycin (Fig. 4D and E). These results suggested the presence of human T cells with normal function in the hu-HSC NOG/HLA-DR4/I-A$\beta^{-/-}$ mice. However, the magnitude of T-cell proliferation was not as robust as that of human T cells from normal healthy donors (Fig. 4D), and the amount of human IL-2 in the culture supernatants was not as high as that produced by normal human T cells (data not shown).

In the present study, using the novel mouse strain NOG/HLA-DR4/I-A$\beta^{-/-}$, we demonstrated that human lymphocytes that developed *in situ* in the humanized mice caused human humoral immune responses in an HLA-DR-restricted manner in cases that used HLA-matched HSC for transplantation. This is a significant advance in humanized mouse technology, as there has yet to be a reliable model in which properly functioning adaptive human immune responses occur without the need for xenotransplantation of human tissues (e.g. fetal liver and thymus in the BLT model) (23).

It has been speculated that the mismatch between the mMHC II responsible for the positive selection of human T cells in the mouse thymus and the HLA II expressed by human B cells in the periphery is the major obstacle to inducing functional human adaptive immune responses in conventional humanized mice (17). Our results demonstrated that this problem was overcome, even if partially, by the introduction of HLA II and the elimination of mMHC II. Recently, Danner *et al.* reported that antigen-specific human IgG was produced in NSG/HLA-DR mice that expressed a human/mouse chimeric molecule, in which the peptide-binding domain of the mouse I-Eβ chain was substituted with the corresponding domain of HLA-DRB1:0401 to mimic the structure of an HLA-DR4 molecule (24, 25). Because they used I-A sufficient NSG mice, the elimination of mMHC II was not necessary for the elicitation of human immune responses. However, it is noteworthy that the I-A sufficient NOG/HLA-DR4/I-A$\beta^{-/-}$ mice in our mouse colony did not induce human IgG responses (data not shown). One plausible explanation for these discrepant results is that mMHC II still played a dominant role in the positive selection of human T cells in I-A$^{+/-}$ mice because of the abundance of mMHC II^{+} TEC. This could render the size of the T-cell repertoire restricted by HLA II too small to induce detectable human immune responses. If this is the case, the elimination of mMHC II is critical to maximize the HLA-restricted T-cell repertoire.

Matching the HLA-DR haplotypes of the recipient NOG/DR4 transgenic mouse and donor HSC significantly influenced human T-cell homeostasis. In particular, HLA-matched reconstitution resulted in a large T_{EM} population, suggesting the HLA-dependent rapid proliferation of human T cells. Considering the extremely lymphopenic environment in NOG mice, the expansion of T_{EM} is reminiscent of lymphopenia-induced proliferation of T cells, which is a well-known phenomenon typically seen when a small number of T_{naive} are seeded into chronically lymphopenic environments (e.g. *RAG* gene deficiency or *scid* mutation) (20, 26). Thus, it is possible that a few human thymic immigrants proliferated to restore the T-cell compartment in NOG mice. This mechanism would explain the higher frequency and higher number of CD4^{+} T cells in the HLA-matched HSC group, compared with the mismatched group, at early time points. Although the same mechanism would also regulate the relatively slower increase of T cells in the HLA-mismatched group, in this case, mouse DC and Mϕ in NOG/DR4/I-A$^{-/-}$ mice predominantly stimulate the T cells, whereas both mouse and human APC would stimulate T cells in the HLA-matched HSC group. The difference in the abundance of APC is one reason for the difference in T-cell homeostasis between the HLA-matched and HLA-mismatched groups.

We previously demonstrated that human T cells in conventional hu-HSC NOG mice had extremely low proliferative capacity in response to antigenic stimulation (17). The accumulation of T_{EM} and the rapid decrease of T_{naive}, which have the largest capacity for proliferation, may be one possible explanation. Indeed, when the frequency of T_{naive} was increased by the transplantation of fetal thymic lobes from NOD mice into the renal capsules of NOG mice, human T cells showed strong proliferation in response to *in vitro* stimulation that was comparable to that of human T cells from healthy human adults (data not shown). This result suggests that high numbers of T_{naive} in NOG/DR4/I-A$^{-/-}$ mice will enable the augmentation of immune responses. There are two major mechanisms that regulate the size of the T_{naive} pool: the supply from the thymus (27, 28) and homeostatic proliferation in the periphery (29, 30). To enhance the function of the thymus (27), transgenic expression of keratinocyte growth factor or flt-3 ligand in TEC, which can enhance the regeneration of TEC after irradiation, should be tested (31,32,33,34,35). Regarding the homeostasis in the periphery, reconstitution of secondary lymphoid organs in NOG mice, which have a significant deficiency of lymph nodes (LN) (36,37,38), is important, as IL-7, a survival factor for T_{naive}, is provided by LN-resident reticular fibroblastic cells (39).

Humanized mice are an excellent tool with which to study human immunology. The reconstitution of a functional human adaptive immune system in hu-HSC NOG/HLA-DR4/I-A$\beta^{-/-}$ mice offers unique opportunities to test and utilize human immunity. For example, the capability to produce antigen-specific IgG in hu-HSC NOG/HLA-DR4/I-A$\beta^{-/-}$ suggests that this model has great potential for generating mAbs against various exogenous substances, such as viral or bacterial proteins. Such mAbs could work as therapeutic drugs for prevention of infection or allergy (18, 19). Along with further improvements of humanized mouse technologies, e.g. introduction of human cytokine genes (40,41,42), this mouse model will contribute to the development of new therapeutic strategies for human disease.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Disclosure

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